Stress induces tRNA cleavage by angiogenin in mammalian cells

Hanjiang Fu1, Junjun Feng1, Qin Liu, Fang Sun, Yi Tie, Jie Zhu, Ruiyun Xing, Zhixian Sun, Xiaofei Zheng *
Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, People’s Republic of China

Abstract

tRNAs play a central role in protein translation, acting as the carrier of amino acids. By cloning microRNAs, we unexpectedly obtained some tRNA fragments generated by tRNA cleavage in the anticodon loop. These tRNA fragments are present in many cell lines and different mouse tissues. In addition, various stress conditions can induce this tRNA cleavage event in mammalian cells. More importantly, angiogenin (ANG), a member of RNase A superfamily, appears to be the nuclease which cleaves tRNAs into tRNA halves in vitro and in vivo. These results imply that angiogenin plays an important physiological role in cell stress response, except for the known function of inducing angiogenesis.

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1. Introduction

tRNAs play a key role in protein synthesis. They provide adaptors for the process in which triplet codons are translated into amino acids, and the vehicles which transport amino acids to ribosome [1]. Past researches on tRNAs mainly focus on their structure, maturation and the correle function in protein synthesis, and little is known about their degradation process. Actually, many factors may lead to the degradation of tRNAs, including the mutation of itself, the inactivation of tRNAs processing or modifying enzyme [2,3].

Many genes responsible for tRNA modifications in the anticodon region play a major role in translation or growth [4]. By contrast, the numerous tRNA modifications outside of the anticodon region have long been considered ancillary. However, Alexandrov et al. [5], have reported that the mutation of any of the three enzymes which are among several non-necessary base-modifying enzymes outside the anticodon loop of tRNAs results in Val-tRNA ACC lacking certain pairs of tRNA modifications, these tRNAs are quickly degraded, and thus lead to temperature-sensitive growth. This rapid tRNA degradation pathway acts on Val-tRNA ACC as fast as for some mRNA species, which demonstrates a critical role of non-essential modifications for tRNA stability and cell survival [5].

Base mutation can also affect the stability of tRNA molecules. Keller et al. [6] have verified that ΔU13 mutant of Ala-tRNA can be polyadenylated by Trf4 complex, subsequently degraded by exosome, whereas none activity was observed with native Ala-tRNA. These results and additional experiments with other tRNA substrates suggest that the Trf4 complex can discriminate between native tRNAs and molecules that are incorrectly folded. There are kinds of mechanisms to ensure the fidelity of macromolecular duplication, transcription and translation in organismic gene expression system, but mutants with mutated base still unavoidably generate. The production of these RNA mutants with mutated base represses translation and thereby severely effects cellular normal metabolism [6]. The tRNA quality control mechanism of tRNAs in cells can remove the tRNAs containing base mutation in time, and so maintains cellular normal metabolism and growth.

All above are about the degradation mechanism of abnormal tRNAs, but the degradation pathway of normal mature tRNAs remains poorly elucidated. By cloning microRNAs from human fetus hepatic tissue [7], we unexpectedly obtained some cleaved tRNA sequences. In this work, we demonstrate that this tRNA cleavage event in mammalian cells is induced by various stress conditions, such as nutrition deficiency, hypoxia and hypothermia. More importantly, we reveal that angiogenin (ANG), a ribonuclease in the RNase A superfamily, appears to be the nuclease which cleaves tRNAs into tRNA halves.

2. Materials and methods

2.1. Cell culture and exposure condition

HepG2, HeLa and other cell lines were grown in DMEM containing 10% FBS and penicillin/streptomycin. For nutrition starvation...
studies, cells were washed and medium were replaced with Dulbecco’s PBS for the indicated times [8]. For serum starvation studies, cells were washed and medium were replaced with serum-free DMEM for the indicated times [9]. For γ-irradiation exposure group, cells were exposed to a final dose of 8Gy or 10Gy (60Co) [10]. For hypoxia treatment, cells were incubated with DMEM containing 100 μM CoCl2 for 1 h or 12 h at 37 °C [11]. For hypothermia treatment, cells were incubated at 16 °C for 40 min, and then incubated at 37 °C for 30 min. For heat shock treatment, cells were incubated at 42 °C for 40 min, and then incubated at 37 °C for 30 min [12].

2.2. Tissue starvation

Fresh tissues of mouse liver and heart were kept in PBS buffers for 30 min, 1 h, 3 h, 5 h, 7 h and 12 h at room temperature, respectively, and then total RNAs were extracted.

2.3. RNA extraction and cloning

Total RNA was extracted from the cultured cells by using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Small RNA (<200 nt) was extracted from cells and human fetal liver tissue using mirVana™ miRNA Isolation kit (Ambion, Austin, TX) according to the manufacturer’s instructions. The concentration of RNA was measured by the UV absorbance at 260 nm. RNA cloning was performed according to the method described previously [7].

2.4. RNA sequence Computational analyses

RNA sequences were subjected to BLAST analyses against the human genome (http://www.ncbi.nlm.nih.gov/blast).

2.5. Expression vector construction and transfection

The angiogenin coding sequence was amplified by polymerase chain reaction (PCR) from HepG2 cDNA with primers 5’-GTCAA- GCTTCTGTGTTGGAAGAGATGGTGATG-3’ and 5’-CACCTCGAGCCGCC TGGTATCGGACGACCGA-3’ and then the PCR product was cleaved with EcoRI and Xhol and ligated into EcoRI/Xhol-cut pcDNA3.0. The resulting plasmid was named pcDNA-ANG. DNA transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Total RNAs and proteins were prepared 48 h after transfection.

2.6. siRNAs and transfection

The siRNAs were synthesized by GeneChem (Shanghai, China). The target sequences of angiogenin siRNA were: (1) AGCUUGUUGG UUGCUGUJUGA; (2) CCAAAGAAUAUAGAAGCUU; and (3) AGCGCAUCUGUAAGAAAACA. The sequence of negative control was UUAAGUAGCUUGGUJUGA. siRNAs transfection in HepG2 cells were performed with Lipofectamine 2000 (Invitrogen) according to its protocol. In brief, cells were cultured in 6-well plate to 40% confluence. For each well, 5 μl siRNA (20 μM) was added into 250 μl Opti-MEM medium, 5 μl of Lipofectamine 2000 into 250 μl Opti-MEM medium, and then mixed siRNA with Lipofectamine 2000. The mixture was added to cells and incubated for 6 h before replacing the medium. Total RNAs and proteins were prepared 48 h after transfection.

2.7. Northern blot

Northern blot was performed according to the method described previously [7]. The probes used for Northern blot were 5’ end of Gly-tRNA: 5’-GCAGGCCGAATCTTACCACCTGAAACCAAT GC-3’; 3’ end of Gly-tRNA: 5’-GCATTGGCCAGAATCGAAGCCGG-3’; 5’ end of Val-tRNA: 5’-GUUUCGGGUGUGUAGGAUGUAAUCAG GUUCCGC-3’; 5’ end of Met-tRNA: 5’-AGGCACTTCGGTCTGCC CACTCCTGT-3’; 5’ end of Arg-tRNA: 5’-GGGCCAGTTGCGCAATGG ATAAACGCTT-3’; 5’ end of Tyr-tRNA: 5’-TCCGCTCTACCAGCTGACC TATCGAAG-3’.

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was used to confirm the siRNA-mediated knock-down of angiogenin mRNA. Reverse transcription was performed according to the protocol of Imprro-Il Reverse Transcriptase (Promega). qPCR was performed as described in the method of SYBR premix Ex Taq (TaKaRa, Dalian, China) with IQ5 (Bio-Rad) supplied with analytical software. GAPDH mRNA levels were used for normalization. Sequences of oligonucleotide primers for qRT-PCR were: angiogenin, 5’-CACCTCGAGCCGCCATCA-3’ and 5’-TCTCTGTGAGGGTTTCCATTC-3’. GAPDH, 5’-TCAGTTGGGACGAGCAGAGC-3’ and 5’-TGCTTGACGACAAATTTGTT-3’.

2.9. RNA cleavage reaction in vitro

For RNA cleavage reaction, incubation mixtures contained 20 μg of total RNA extracted from HepG2 cells, 1 μM angiogenin (90%, Cancer Institute, Chinese Academy of Medical Science, Beijing, China), 30 mM HEPES, pH 6.8, 30 mM NaCl, 0.001%BSA. Incubations were carried out at 37 °C for 10 min, 30 min, 1 h or 2 h. Then the cleaved products were recovered by phenol/chloroform extraction followed by ethanol precipitation [13].

2.10. Western-blot analysis

Protein extracts were prepared by a modified RIPA buffer with 0.5% sodium dodecyl sulfate (SDS) in the presence of proteinase inhibitor cocktail (Complete mini, Roche). Polyacrylamide gel electrophoresis, tank-based transfer to Immobilon Hybond-C membranes (Amersham Biosciences), and immunodetection were performed with standard techniques. Antibodies against ANG (sc-1408, Santa Cruz), and β-actin (sc-1616-R, Santa Cruz) were used in Western-blot analysis in accordance with the manufacturer’s instruction. Signals were visualized with SuperSignal™ West Pico chemoluminescent substrate (Pierce) by exposure to films.

3. Results and discussion

3.1. Identification of cleaved mammalian tRNA fragments

When we tried to clone microRNAs from human fetal hepatic tissue, we unexpectedly have obtained a distinct population of 31–38nt cleaved tRNA fragments [7]. These tRNA fragments correspond to five different kinds of tRNAs (Table 1). Intriguingly, they do not appear to result from the random degradation of tRNAs, but instead correspond to ‘halves’ of tRNAs located either 5’ or 3’ of the anticodon sequence. None of these tRNA fragments are generated from fully mature tRNAs rather than from nascent tRNA transcripts. In other words, the observed tRNA halves were derived from cleavage within the anticodon loop of mature tRNAs by specific endonuclease.

To determine whether these tRNA fragments were products of specific cleavage or random degradation, we analyzed individual tRNA by Northern blot. Small RNAs (<200nt) were extracted from fetal liver, HepG2, BEL-7402, HeLa, A549, HEK-293 and Cos7 cell
We detected the tRNA fragments with probes complementary to the 5' end and 3' end of Gly-tRNAGCC, respectively. As shown in Fig. 1, not only intact tRNAs, but also both 5' end and 3' end of Gly-tRNAGCC were detected in all tissue and cell lines. The tRNA halves detected in fetal liver were much more than those of other cell lines. This revealed that the tRNA fragments we cloned were products of specific cleavage of intact tRNAs. Although we have not cloned 3' end sequence of Gly-tRNAGCC, it was detected by Northern blot suggesting that the kinds of tRNA halves were more than those of we cloned.

### 3.2. Stress conditions induce the production of tRNA halves

Given that fetus hepatic tissue had been kept outside the body for a few hours before RNA was extracted, we selected mouse liver and heart tissues to analyze the cause of tRNA halves generation. First, we extracted total RNAs from liver and heart as soon as the mouse was killed, and the remaining parts of liver and heart were kept in PBS buffer at room temperature. Then total RNAs were extracted from these tissues at different time point, such as 30 min, 1 h, 3 h, 5 h, 7 h and 12 h, respectively. These total RNAs were analyzed with the probe complementary to the 5' end of Val-tRNA. Northern blot analysis indicated that the levels of tRNA halves in normal liver tissue and heart tissue were low, but they increased when these tissues were kept in PBS, and the levels of tRNA halves reached to maximum at 3 h (Fig. 2). These results suggest that the generation of tRNA halves may be induced by nutrition deficiency.

To investigate whether this is the same case for cells cultured in vitro, total RNAs were extracted from HepG2 cells cultured in PBS or DMEM without serum. The tRNA halves derived from Gly-tRNA, Val-tRNA, Met-tRNA, Arg-tRNA and Tyr-tRNA were analyzed by Northern blot. The result revealed that nutrition deficiency could induce the production of multiple tRNA halves in cells cultured in vitro indeed (Fig. 3). 5' half fragment of Met-tRNA has not been cloned by our strategy, but it was detected by Northern blot (Fig. 3c), which suggested that more kinds of tRNAs than those we cloned could be cleaved into tRNA halves under certain conditions. Lee et al. found that tRNA halves generated in *tetrahymena thermophila* cells lacking essential amino acids, and that the addition of these essential amino acids could inhibit the production of tRNA halves [14]. However, tRNA halves could generate in human tumor cells either cultured in PBS or DMEM (rich of essential amino acids) without serum (Fig. 3). That is to say, only essential

### Table 1

Summary of cleaved tRNA fragments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>No. of clones</th>
<th>Size (nt)</th>
<th>5' or 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-tRNA&lt;sup&gt;GGG&lt;/sup&gt;</td>
<td>GCGGGTGTTCTGCTGCTGGGCTGCCTGCTGCC</td>
<td>19</td>
<td>32–35</td>
<td>5</td>
</tr>
<tr>
<td>Gly-tRNA&lt;sup&gt;CCC&lt;/sup&gt;</td>
<td>GCGGGTGTTCTGCTGCTGGGCTGCCTGCTGCC</td>
<td>4</td>
<td>31</td>
<td>5</td>
</tr>
<tr>
<td>Val-tRNA&lt;sup&gt;ACC&lt;/sup&gt;</td>
<td>GTTGGTCTCCTTCTGCTGCTGGGCTGCCTGCTGCC</td>
<td>11</td>
<td>32–33</td>
<td>5</td>
</tr>
<tr>
<td>Glu-tRNA&lt;sup&gt;ACC&lt;/sup&gt;</td>
<td>TGGTGTTCTGCTGCTGCTGGGCTGCCTGCTGCC</td>
<td>7</td>
<td>33–35</td>
<td>5</td>
</tr>
<tr>
<td>Arg-tRNA&lt;sup&gt;GGG&lt;/sup&gt;</td>
<td>GCGGGTGTTCTGCTGCTGGGCTGCCTGCTGCC</td>
<td>2</td>
<td>38</td>
<td>3</td>
</tr>
</tbody>
</table>

Sequences listed represent the longest sequence of each kind of tRNA halves identified in this study.

- **No. of clones.**
- **tRNA fragments are 5' or 3' ends of intact tRNA.

![Fig. 1](image1.png)

**Fig. 1.** Detection of the 5' and 3' halves of Gly-tRNA by Northern blot. (a) tRNA halves were detected by the probe complementary to the 5' sequence of Gly-tRNA<sup>GGG</sup> and (b) tRNA halves were detected by the probe complementary to the 3' sequence of Gly-tRNA<sup>GGG</sup>. Total RNAs were extracted from fetal liver tissue and diverse cell lines, and analyzed by Northern blot.

![Fig. 2](image2.png)

**Fig. 2.** tRNA halves investigated by Northern blot in starvation tissues. (a) tRNA halves were detected by the probe complementary to the 5' sequence of Val-tRNA<sup>ACC</sup> in liver tissue and (b) tRNA halves were detected by the probe complementary to the 5' sequence of Val-tRNA<sup>ACC</sup> in heart tissue. Total RNAs were extracted from mouse liver and heart tissue at different time point, and analyzed with 5.8S specific probe of Val-tRNA<sup>ACC</sup>. As a loading control, 5.8S RNAs were stained with ethidium bromide (EtBr).
amino acids can not inhibit the production of tRNA halves in mammalian cells.

As shown in Fig. 3e, not all types of tRNAs were cleaved into tRNA halves under the condition of nutrition deficiency. We could detect intact Tyr-tRNAGTA but not its cleaved fragments from cells treated with both PBS starvation and non-serum starvation (Fig. 3e). That means the production of its tRNA halves could not be induced by nutrition deficiency. However, the level of Tyr-tRNAGTA decreased significantly when cells were cultured in PBS for 7 h. The length of Tyr-tRNAGTA is ~90nt, but the lengths of other tRNAs such as Gly-tRNA, Val-tRNA and Met-tRNA, are ~70nt. That suggests the structure of Tyr-tRNAGTA may be different from other tRNAs, and it might be cleaved at other sites instead of anticodon loop.

To further analyze the reasons of tRNA halves generation, HepG2 cells were treated with several stress conditions such as heat shock, hypothermia, hypoxia or irradiation. Northern blot analysis showed the levels of cleaved Val-tRNA fragment increased in cells treated with PBS starvation for 30 min, heat shock at 42 °C, hypothermia treatment at 16 °C, hypoxia by CoCl₂ for 1 h or hypoxia by CoCl₂ for 12 h (Fig. 4). And hypothermia treatment had the most significant effects on the production of tRNA halves. However, irradiation had no distinct effects on the production of tRNA halves. These results reveal that not only nutrition deficiency but also multiple stress conditions can trigger tRNA cleavage and lead to the generation of tRNA halves in mammalian cells.

3.3. Angiogenin can cleave tRNAs in the anticodon loop in vitro

The tRNAs can be cleaved in the anticodon loop and processed into tRNA halves under the conditions of nutrition deficiency or stress. This cleavage may be catalyzed by a site-specific nuclease that recognizes specific tRNA structure. Anticodon loop cleavage of tRNAs has been observed in prokaryotes, the prokaryotic plas-
mid-encoded Colicins and the anticodon nuclease PrrC target tRNAs for cleavage in the anticodon loop in response to stress-induced signals and T4 phage infection, respectively [15,16]. Unlike the global tRNA cleavage that we have observed here, these molecules can only act on several specific tRNAs. In addition, BLAST searches suggest that there are no similar proteins in human cells (data not shown). Thus, other RNases should be responsible for cleavage of tRNAs induced by stress condition.

Saxena et al. have reported that angiogenin (ANG), a member of RNase A superfamily, can degrade tRNAs and inhibit protein synthesis when injected into Xenopus oocytes [17]. To verify whether tRNAs can be cleaved into tRNA halves by angiogenin, 20 μg total RNAs were incubated with 1 μM recombinant angiogenin in vitro at 37°C for 10 min, 30 min, 1 h and 2 h, respectively. Northern blot results showed that most of the intact Val-tRNA was cleaved into short tRNA halves after 10 min (Fig. 5). And the longer total RNAs were incubated, the more intact tRNAs were cleaved. When total RNAs were incubated with angiogenin for 2 h, almost all intact tRNAs and cleaved tRNA halves were degraded. These results suggest that the production of tRNA halves initiates a complete degradation pathway for targeted tRNAs.

3.4. Angiogenin cleaves tRNAs into tRNA halves in vivo

To test whether angiogenin could cleave tRNAs in cultured mammalian cells, HeLa cells were transiently transfected with the plasmid expressing angiogenin. Cellular total RNAs were extracted 48 h after transfection and analyzed by Northern blot using probes targeted to 5' ends of Met-tRNA, Tyr-tRNA and Val-tRNA, respectively. As shown in Fig. 6, 5' ends tRNA halves of Met-tRNA and Val-tRNA were detected in angiogenin over-expressing cells but not in negative control samples. However, we detected no half fragment of Tyr-tRNA in angiogenin over-expressing cells, which coincided with our previous results that its tRNA halves were not detected in starving cells. These results suggest that angiogenin is probably the endonuclease for producing tRNA halves in vivo.

In order to analyze the role of endogenous angiogenin in production of tRNA halves, HepG2 cells were transfected with chemically synthesized siRNAs against angiogenin. All three siRNAs can reduce the mRNA levels of angiogenin, and siANG-1 has significant inhibition effect on the expression of angiogenin (Fig. 7a and b), so it was used in the following experiment. HepG2 cells were transfected with siANG-1 and control siRNA, respectively, 48 h after transfection, the medium was replaced with PBS for 30 min. Then the total RNAs were extracted and analyzed for the produc-
tion of Val-tRNA halves. Depletion of angiogenin in cells cultured with normal DMEM medium had no significant effects on the production of tRNA halves (Fig. 7c). But comparing with negative siRNA control, siANG-1 inhibited the production of tRNA halves in cells treated with PBS (nutrition deficiency), which suggests that angiogenin is essential for the production of tRNAs halves in starved mammalian cells. Angiogenin is a member of the RNase superfamily, and plays an important role in eliciting new blood vessel growth, angiogenesis [18]. By regulating angiogenesis, angiogenin is closely related to tumor growth and progression, and even its aggressiveness [19]. Site-directed mutagenesis studies have shown the intact RNase active site is crucial for angiogenesis [20]. However, its intracellular natural substrate is not clear. Here we have found that tRNAs can be cleaved into tRNA halves by angiogenin in vitro and in vivo (Figs. 5 and 6), suggesting tRNAs may be natural substrates of angiogenin in mammalian cells. These tRNA halves have accumulated under various stress conditions (Fig. 4), however, no significant angiogenin protein levels alteration was detected (Fig. S1). That might because tRNA cleavage by angiogenin occurs preferentially for deacylated tRNAs lacking 3′-CCA. A fraction of deacylated tRNA may lose 3′terminal CCA nucleotides under stress conditions, and these truncated tRNAs accumulate transiently [14]. Therefore, tRNA halves cleaved by angiogenin accumulate without angiogenin protein levels alteration.

Although tRNA halves have been identified in various species such as Streptomyces coelicolor [21], Tetrahymena thermophila [14], Caenorhabditis elegans [22] and mammalian cells, the biological function of tRNA cleavage is still unclear. It is conceivable that the tRNA cleavage could serve as a mechanism to eliminate redundant tRNA molecules, especially those uncharged and/or 3′-truncated tRNAs. Targeting redundant tRNAs for degradation may save more essential nutritive material to maintain longer term survival under nutrient-poor conditions. Another possibility is that cleaved tRNA halves might act as signaling molecules to regulate genes. For example, miRNAs of influenza virus and HIV-1 can be cleaved by the RNases (RNase P and RNase Z) with external guide sequence (EGS), which is an RNA similar to tRNA halves form and plays its role as a guide to specify its target RNAs [23,24]. These RNAs may use the cleaved product of tRNA identified here as a guide to regulate the expression of target genes. The ribonucleolytic activity of angiogenin is crucial for its angiogenic activity [20], here we demonstrate that tRNAs are natural substrates of angiogenin in mammalian cells. Then it is also possible that tRNA cleavage by angiogenin plays a role in the angiogenesis process. All of the above hypotheses, based on our novel data, await future experimental validation.

In summary, our data show that tRNA halves are commonly found in diverse mammalian cells, and this tRNA cleavage event can be induced by multiple stress conditions. In addition, angiogenin, a member of RNase A superfamily, appears to be the nuclease which cleaves tRNAs into tRNA halves in vitro and in vivo. That is to say, tRNAs are natural substrates of the ribonucleolytic activity of angiogenin, although the biological function of these tRNA halves remains to be elucidated.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.febslet.2008.12.043.

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